

THE MOUSE/HUMAN CHIMERIC MONOCLONAL ANTIBODY cA2 NEUTRALIZES TNF IN VITRO AND PROTECTS TRANSGENIC MICE FROM CACHEXIA AND TNF LETHALITY IN VIVO

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The pleiotropic cytokine tumour necrosis factor- α (TNF) is thought to play a central role in infectious, inflammatory and autoimmune diseases. Critical to the understanding and management of TNF-associated pathology is the development of highly specific agents capable of modifying TNF activity. We evaluated the ability of a high affinity mouse/human chimeric anti-TNF monoclonal antibody (cA2) to neutralize the in vitro and in vivo biological effects of TNF. cA2 inhibited TNF-induced mitogenesis and IL-6 secretion by human fibroblasts, TNF-priming of human neutrophils, and the stimulation of human umbilical vein endothelial cells by TNF as measured by the expression of E-selectin, ICAM-1 and procoagulant activity. cA2 also specifically blocked TNF-induced adherence of human neutrophils to an endothelial cell monolayer. Receptor binding studies suggested that neutralization resulted from cA2 blocking of TNF binding to both p55 and p75 TNF receptors on the cells. In vivo, repeated administration of cA2 to transgenic mice that constitutively express human TNF reversed the cachectic phenotype and prevented subsequent mortality. These results demonstrated that cA2 effectively neutralized a broad range of TNF biological activities both in vitro and in vivo.

Tumour necrosis factor- α (TNF) is a cytokine that exhibits a pleiotropic spectrum of activities, with receptors found on virtually all cell types examined.^{1,2} The natural functions of TNF are thought to include modulation of the host immune and inflammatory response to a variety of infectious, malignant and autoimmune conditions as part of a complex regulatory mechanism in which numerous other cytokines participate.³ While initial TNF expression in response to infection or injury would be considered beneficial, excessive production, usually by activated monocytes and macrophages, can result in significant pathological changes.

TNF has been implicated as the primary mediator in bacterial sepsis since it is the first proinflammatory cytokine detected in primate and human volunteer studies where serum cytokine levels were measured after administration of endotoxin.⁴ Administration of TNF to rodents and dogs induced a profile of pathophysiological changes and lethality similar to that seen after endotoxin challenge.^{5,6} Neutralizing antibodies to TNF have been shown to prevent physiological changes and death in animal models of endotoxin and bacteremia.⁷⁻⁹

There are also a number of autoimmune disorders in which TNF appears to play a significant role,¹⁰⁻¹² but the evidence is most persuasive in rheumatoid arthritis.¹³⁻¹⁶ Rheumatoid arthritis (RA) is characterized by a chronic inflammation of the synovial lining of multiple joints. Synovial cells proliferate along with infiltrating inflammatory cells and vascularity increases markedly. Ultimately, the release of degradative enzymes results in irreversible erosion of the bone and cartilage components of the joint.^{13,14} Levels of TNF are not only elevated in synovial fluid from the joints of RA patients^{17,18} but cells from the synovial fluid continue to produce TNF when cultured in vitro.¹⁹ Perhaps the most direct evidence that TNF plays a pivotal role in the development of arthritis was obtained by the constitutive expression of TNF in transgenic mice.²⁰ Such mice develop chronic inflammatory polyarthritis at a specific age (depending on the transgenic mouse line)

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and with a 100% phenotypic penetrance. Thus, TNF appears to be an attractive target and an antibody which efficiently neutralizes human TNF may be an effective treatment in RA.^{20, 21}

We have previously described the construction of a chimeric mouse/human IgG₁ monoclonal antibody which binds to TNF.²² This antibody, designated cA2, exhibited high affinity and specificity for TNF and neutralized both recombinant and natural human TNF in the standard assay of TNF cytotoxicity. cA2 was also shown to be highly species-specific, neutralizing TNF from only humans and chimpanzees. The safety and potential efficacy of cA2 in treating autoimmune disorders is currently being evaluated in human clinical trials.^{23, 24}

In this study we examined the effect of cA2 on the in vitro biological activity of TNF and on TNF-receptor interactions. The ability of cA2 to modulate the in vivo activity of TNF was also investigated using an established transgenic mouse line that develops a cachectic phenotype and accelerated mortality due to constitutive expression of human TNF.²⁵

RESULTS

Effect of cA2 on TNF-stimulated FS-4 fibroblasts

The fibroblast cell line FS-4 proliferates and se-

cretes IL-6 in response to recombinant human TNF- α (TNF).^{26, 27} As shown in Table 1, TNF at concentrations ranging from 0.3 to 7.5 ng/mL induced FS-4 fibroblasts to produce IL-6 levels ranging from 1300 to 2500 pg/mL. When cA2 was added to the medium at the same time as TNF, the induction of IL-6 was completely blocked at the 0.3 and 1.5 ng/mL TNF dose levels, and only a small amount of IL-6 was detected (300 pg/mL) at the highest (7.5 ng/mL) TNF dose. The control antibody had no effect.

Similarly, cA2 was shown to block the mitogenic effect of TNF on FS-4 fibroblasts (Table 2). When cA2 was added to the culture medium with TNF, proliferation was blocked at all three TNF levels tested (0.1, 0.5 and 2 ng/mL). The control IgG had no effect on the TNF-induced proliferation of FS-4 fibroblasts. A significant difference ($P \leq 0.03$ at all concentrations) was demonstrated between the cA2 and control groups using the nonparametric Wilcoxon test. In additional experiments, cA2 also completely inhibited mitogenesis at TNF concentrations up to 8 ng/mL (data not shown).

Effect of cA2 on TNF-stimulated human umbilical vein endothelial (HUVE) cells

HUVE cells produce a procoagulant activity (PCA) when exposed to TNF which appears to be related to

TABLE 1. Neutralization of TNF-induced IL-6 secretion by fibroblasts

Antibody	IL-6 production (pg/mL)			
	No TNF	0.3 ng/mL TNF	1.5 ng/mL TNF	7.5 ng/mL TNF
None	<200	1360	2000	2560
Control	<200	1600	1960	2160
cA2	<200	<200	<200	300

Recombinant human TNF, preincubated with or without 4 μ g/mL cA2 or control antibody, was added to cultures of FS-4 human fibroblasts. After 18 h incubation, IL-6 levels in the supernatant were determined by immunoassay.

TABLE 2. Neutralization of TNF-induced mitogenesis in fibroblast cultures

Antibody	Cell Density (OD 630 nm $\times 10^3$)			
	No TNF	0.1 ng/mL TNF	0.5 ng/mL TNF	2.0 ng/mL TNF
None	36 \pm 1	50 \pm 1	55 \pm 2	63 \pm 1
Control	35 \pm 2	49 \pm 1	54 \pm 1	63 \pm 1
cA2	39 \pm 1	37 \pm 1	39 \pm 1	41 \pm 2

FS-4 human fibroblasts were seeded in 96-well plates. Recombinant human TNF, preincubated with or without 4 μ g/mL cA2 or control antibody, was added to the cultures and cell density was determined by staining 5 days later. Data represent the mean (\pm standard deviation) of quintuplicate wells.

tissue factor upregulation.²⁸ After the cells are lysed by freezing and thawing, PCA can be detected by measuring the clotting time of plasma to which calcium and cell lysate have been added. Figure 1 shows that while a clotting time of about 130 sec is obtained using an unstimulated HUVE lysate, addition of lysate from HUVE cells exposed to 25 ng/mL TNF for 4 h at 37°C shortens the clotting time by 50%. This reduction in clotting time was blocked by the addition of cA2 in the HUVE cell medium during the time of TNF exposure. A final cA2 concentration of 0.37 μ g/mL could completely neutralize TNF-induced PCA while isotype-matched control IgG at 10 μ g/mL had no effect.

TNF also acts on HUVE cells by inducing the cell surface expression of the adhesion proteins E-selectin²⁹ and ICAM-1.³⁰ Peak temporal expression of these surface antigens varies, however they can be individually quantified using specific monoclonal antibodies. The observed levels of TNF-induced E-selectin and ICAM-1 expression on the HUVE cell surface were reduced in a dose-dependent manner by the inclusion of cA2, while control IgG had no effect (Fig. 2). Induction of peak E-selectin expression (measured at 4 h) was fully abrogated by 0.5 μ g/mL of cA2 (Fig. 2A), and complete blocking of peak ICAM-1 expression (at 23 h) required 0.1 μ g/mL of cA2 (Fig. 2B).

In vivo, the expression of E-selectin and ICAM-1 by endothelial cells exposed to TNF results in the binding of circulating neutrophils and eventual extravasation into the surrounding tissues.³¹ In order to determine if cA2 could effectively block the adhesion of neutrophils in an in vitro system, HUVE cell monolayers were first exposed to TNF in the presence and absence of cA2 for 4 h, then exposed to isolated human neutrophils. After washing, the percentage of neutrophils that adhered to the HUVE cells was determined using a

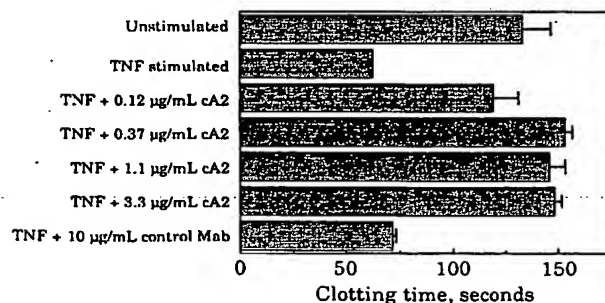


Figure 1. Effect of cA2 on the production of rhTNF-induced procoagulant activity by HUVE cells.

cA2 or control antibody were preincubated 30 min at room temperature with 25 ng/mL rhTNF prior to cell stimulation for 4 h at 37°C. Procoagulant activity was measured by determining the clotting time of recalcified human plasma after addition of the treated HUVE cell lysates. Data shown are the mean of duplicate wells \pm SEM.

neutrophil-specific myeloperoxidase assay. When treated with TNF alone, neutrophil adherence increased 52%, a fourfold increase over unstimulated HUVE cells (Fig. 3). Increasing amounts of cA2 reduced the degree of neutrophil adherence in a dose-dependent manner, while 3.3 μ g/mL of control IgG had no effect. cA2 had no effect on neutrophil adherence to HUVE cells treated with IL-1 or LPS, demonstrating that the inhibitory effect of cA2 is directed specifically at TNF.

The inhibition of TNF-induced neutrophil adherence was also apparent by phase contrast light microscopy (Fig. 4). Neutrophils can be identified by their rounded, highly refractile appearance as opposed to the flat morphology exhibited by the HUVE cell monolayer. In Panel A, unstimulated HUVE cells show few adherent neutrophils compared to HUVE cells stimulated with TNF (Panel B). The presence of control IgG during TNF stimulation (Panel C) had no effect, while the presence of 3.3 μ g/mL of cA2 during

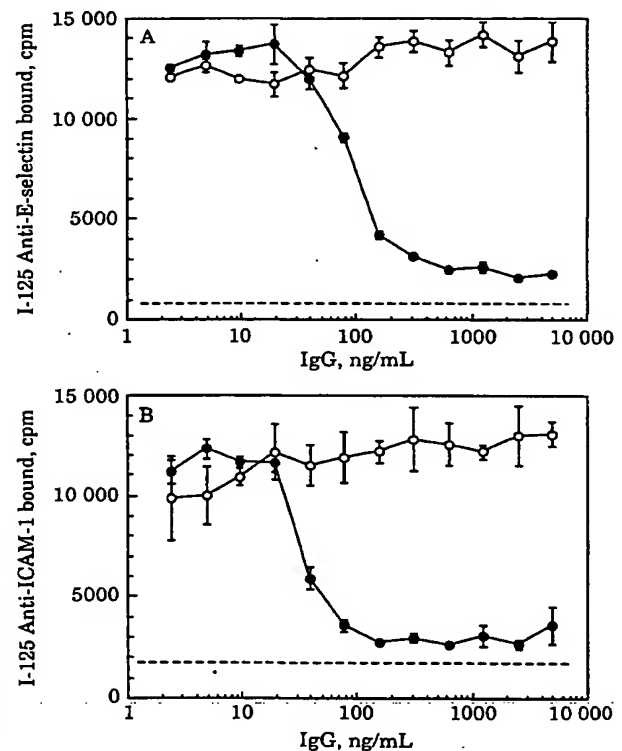


Figure 2. Neutralization of rhTNF-induced HUVE cell E-selectin and ICAM-1 expression by cA2.

Serial dilutions of cA2 (●) or control antibody (○) were mixed with 10 ng/mL of rhTNF and used to stimulate endothelial cell monolayers. E-selectin (Panel A) and ICAM-1 (Panel B) were detected after stimulation for 4 h and 23 h, respectively, using ¹²⁵I monoclonal antibodies specific for each adhesion protein. Data shown are the mean of triplicate wells \pm SEM. The values obtained on unstimulated cells were 736 \pm 65 cpm for E-selectin at 4 h and 1723 \pm 149 cpm for ICAM-1 at 23 h (shown by dashed line).

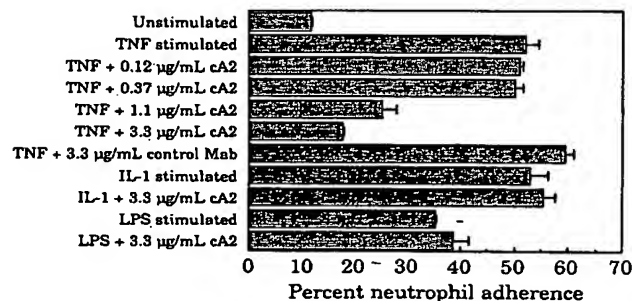


Figure 3. Neutralization of rhTNF-induced human neutrophil adhesion to HUVE cells by cA2.

HUVE cell monolayers (about 3×10^5 cells per well) were stimulated for 4 h with rhTNF (250 ng/mL), IL-1 α (40 units/mL), J5 LPS (10 ng/mL) or medium alone (unstimulated). Incubations were performed in the presence or absence of the indicated concentrations of cA2 or control antibody. After washing, the stimulated HUVE cells were incubated an additional 45 min with human neutrophils (1×10^6 per well), and the number of neutrophils bound to the HUVE cells was determined by the myeloperoxidase assay. The data shown represents the average of triplicate wells \pm SEM.

TNF stimulation (Panel D) markedly reduced the number of adherent neutrophils.

Effect of cA2 on TNF priming of human neutrophils

In vitro, TNF primes human neutrophils to produce superoxide upon subsequent stimulation with the chemotactic peptide f-met-leu-phe (FMLP).³² The ability of cA2 to abrogate the TNF-priming phenomenon is shown in Figure 5. In the absence of cA2, TNF-primed (2 ng/mL) neutrophils produced as much as 40–45 nM superoxide upon stimulation with FMLP. TNF alone induced little or no superoxide production. cA2 was able to reduce the TNF-induced priming phenomenon in a dose-dependent manner, with levels as low as 1 µg/mL able to reduce superoxide production to the level typically seen with unprimed FMLP-stimulated neutrophils (7.85 nM in the experiment shown). Incubation of up to 100 µg/mL of control antibody with TNF had no effect on the subsequent production of superoxide upon FMLP stimulation (data not shown).

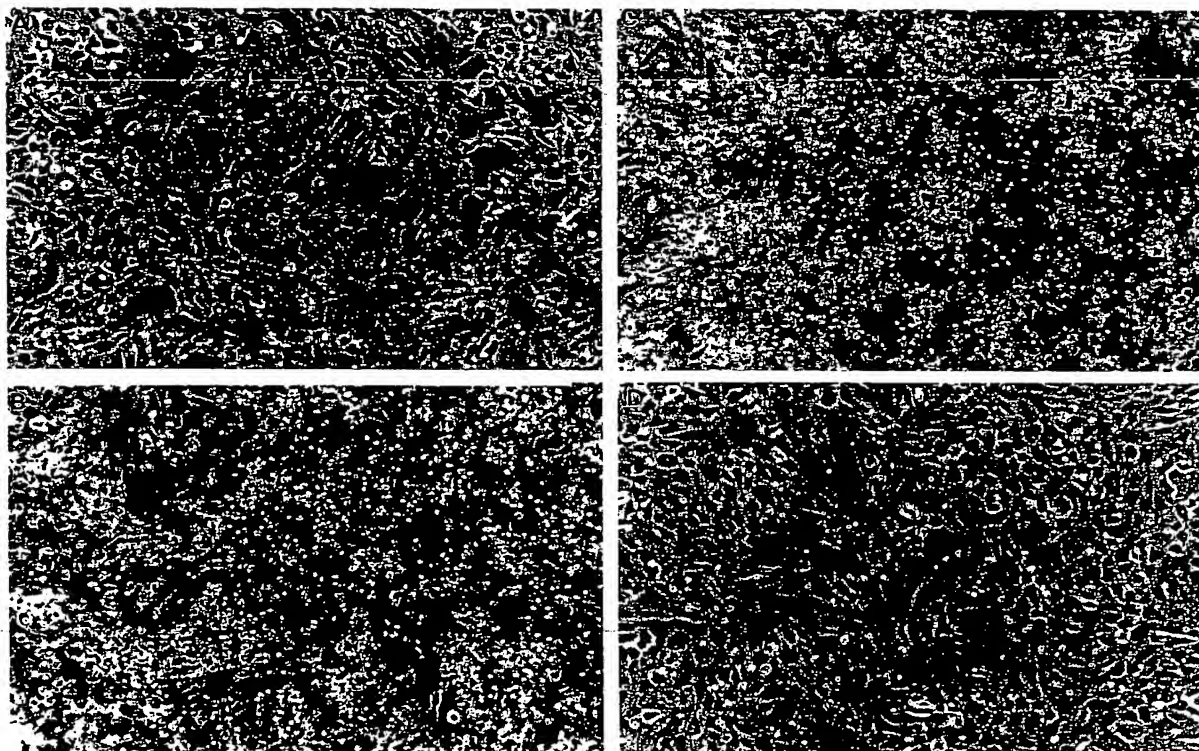


Figure 4. Phase contrast micrographs demonstrating neutrophil adherence to HUVE cells stimulated with rhTNF in the presence and absence of cA2.

HUVE cells were stimulated for 4 h with medium alone (A), 250 ng/mL rhTNF (B), 250 ng/mL rhTNF and 3.3 µg/mL control antibody (C) or 250 ng/mL rhTNF and 10 µg/mL cA2 (D). After stimulation, the ability of human neutrophils to adhere to the HUVE cell monolayer was assessed. Phase contrast micrographs were taken at 100-fold magnification.

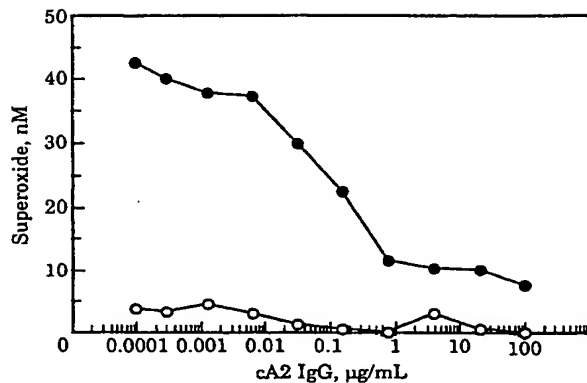


Figure 5. Effect of cA2 on the ability of rhTNF to prime human neutrophils.

Neutrophils (2.5×10^6 cells per mL) were primed with 2 ng/mL rhTNF in the presence of cA2 for 60 min, then the neutrophil activator FMLP (0.1 μ M) (●) or medium (○) was added and the incubation continued for 10 min. Superoxide ion production was detected via the oxidation of cytochrome C. The data represent the average of duplicate wells with the background signal observed in parallel samples containing superoxide dismutase subtracted. Superoxide ion production by unprimed neutrophils exposed to FMLP was 7.85 nM.

Effect of cA2 on TNF binding to receptor

The inhibition of TNF biological activity by cA2 presumably occurs as a result of the ability of cA2 to bind to soluble TNF, thereby inhibiting its interaction with cellular receptors. In order to directly demonstrate this *in vitro*, experiments were performed using both a commercially-available TNF receptor binding assay and recombinant immunoadhesion constructs of the human p55 and p75 TNF receptors. Figure 6 shows that cA2 inhibits the binding of radiolabelled TNF to a preparation of U937 monocytic cell membranes which contain TNF receptors. U937 cells have been previously shown to express both the p55 and p75 TNF receptors.³³ cA2 inhibition was dose-dependent, with 50% inhibition observed at 0.1 μ g/mL.

Similarly, the binding of radiolabelled TNF to recombinant constructs containing the extracellular domain of either the p55 or p75 TNF receptor was inhibited by cA2 (Fig. 7). Inhibition by cA2 was dose-dependent, and 50% inhibition of binding to either receptor construct was evident at about 0.03 μ g/mL cA2.

Effect of cA2 in a transgenic mouse model

Transgenic mice have been generated in which human TNF is constitutively expressed by their T cells.²⁵ These animals show elevated human TNF serum levels (0.01–7 units/mL) and develop a lethal wasting syndrome resulting in 80%–100% mortality at 10–18 weeks

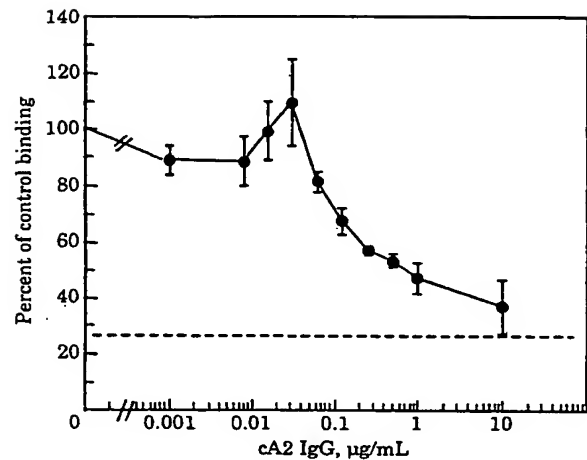


Figure 6. Binding of 125 I-rhTNF to U937 cell membranes in the presence of cA2.

U937 membranes were incubated for 3 h at 0°C with 125 I-rhTNF (45 pM) and the indicated concentration of cA2. 125 I-rhTNF bound to membranes was then separated by filtration and counted. Data are expressed as the mean \pm SEM (three experiments) percent of binding in the absence of cA2 (1100 cpm). Binding of 125 I-rhTNF in the presence of 10 μ g/mL negative control antibody was $96 \pm 1\%$. Binding of 125 I-rhTNF in the presence of 40 nM unlabelled TNF (nonspecific background) was $26 \pm 9\%$ (shown by dashed line).

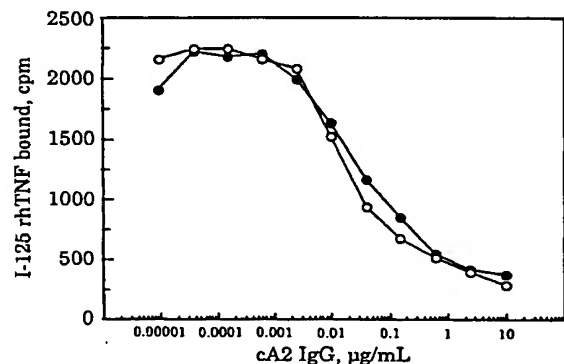


Figure 7. Binding of 125 I-rhTNF to p55 or p75 receptor fusion protein in the presence of cA2.

Serial dilutions of cA2 in 4 ng/mL 125 I-rhTNF were incubated on p55 (●) or p75 (○) receptor-coated microtiter wells for 1 h at 37°C. The data represent the mean of duplicate wells. Binding of 125 I-rhTNF in the presence of 10 μ g/mL negative control antibody was 2270 cpm.

after birth. The ability of cA2 to prevent mortality in the transgenic mouse line Tg211 is shown in the survival curves of Figure 8. There was complete survival (15/15) to the 8 week endpoint in 3 week old animals administered cA2 twice-weekly at doses of 8 or 2 mg/kg. In the group administered the lowest dose of cA2, 0.5 mg/kg, there was a final 93% survival rate (14/15). By contrast,

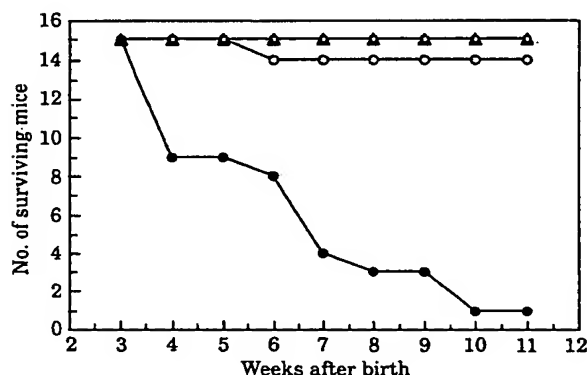


Figure 8. Survival curves of rhTNF transgenic mice treated with 0.5 (○), 2 (▲) and 8 (□) mg/kg cA2 IgG or 8 mg/kg control antibody (●).

Treatments were given twice weekly beginning at 3 weeks of age and continued for 8 weeks.

the control antibody group had a final survival rate of 7% (1/15). The difference between the least protective cA2 group (0.5 mg/kg) and the control group was highly statistically significant, log-rank $P = 0.0001$. The beneficial effect of cA2 was also evident when comparing weight gain between groups. Table 3 shows the average weight for each group at study entry (week 3), at study mid-point (week 7), and at study termination (week 11). Animals in the three cA2 treatment groups showed significant weight gain throughout the 8-week study which averaged 134%. By contrast, the control group showed only a modest (49%) weight gain throughout the 8-week study. Even this modest weight gain in the control group may be an overestimation since the control animals that died during the study may have experienced the least weight gain; however, they were obviously excluded from the later time points.

DISCUSSION

In this study we evaluated the effect of the chimeric anti-human TNF monoclonal antibody cA2 on both the in vitro and in vivo biological activities of TNF. cA2 exhibited potent neutralizing activity against TNF-mediated effects on human fibroblast, neutrophil and endothelial cell cultures. In the bioassays examined, 4 $\mu\text{g/mL}$ of cA2 was sufficient to prevent the biological effects of an optimal challenge dose of TNF. The effects of cA2 were dose-dependent and highly specific. Incubation of cA2 with TNF directly inhibited the binding of TNF to receptor-containing cell membrane preparations and to recombinant constructs of the human p55 and p75 TNF cellular receptors. The neutralizing activity of cA2 could also be demonstrated in vivo, as evidenced by the ability of cA2 to reverse both the phenotype and accelerated mortality seen in transgenic mice that express the human TNF gene. In this case, cA2 efficacy was demonstrated in a chronic disease model by administering the drug over a period of 8 weeks. The potency of cA2 was evidenced by the suppression of mortality and weight loss with cA2 doses as low as 0.5 mg/kg administered twice weekly. These observations support and extend our previous studies which showed cA2 to be a high-affinity, highly-specific, TNF neutralizing antibody.²²

The data presented for cA2 are consistent with the previously reported benefit of anti-TNF therapy in animal models of bacterial sepsis. Sepsis is a complex syndrome and it may be necessary to develop therapeutic modalities which target multiple disease mediators. Although clinical trials using passive anti-TNF immunotherapy are underway,³⁴ no efficacy data have been reported.

How do these findings relate to the potential application of cA2 to human autoimmune disease? In the case of RA, TNF has been implicated as a primary mediator of the chronic inflammation, although other

TABLE 3. Mortality and weight changes in TNF transgenic mice treated with cA2

Group	Study entry (week 3)		Study midpoint (week 7)		Study termination (week 11)	
	Average wt (g)	Number of Survivors	Average wt (g)	Number of Survivors	Average wt (g)	Number of Survivors
8 mg/kg cA2	7.4	15	16.0	15	20.0	15
2 mg/kg cA2	9.3	15	16.9	15	19.6	15
0.5 mg/kg cA2	7.6	14	15.9	14	16.8	14
8 mg/kg control antibody	10.0	4	11.4	1	14.9	1

Three week old Tg211 transgenic mice expressing rhTNF ($n = 15$ per group) were injected twice weekly with cA2 or an isotype-matched control antibody. Weights were measured weekly and represent the average of surviving animals.

inflammatory cytokines are also important.^{16, 35} TNF has been shown *in vitro* to cause proliferation of synovial cells³⁶ as well as fibroblasts²⁶ which could directly contribute to pannus formation and fibrosis, respectively. This correlation is strengthened by the report that TNF is produced by cells at the cartilage-pannus junction³⁷ and is secreted spontaneously by cultured cells from synovial fluid from RA patients.¹⁹ Thus, the ability of cA2 to block TNF-induced mitogenesis of human diploid fibroblasts, as well as their secretion of IL-6, may be relevant to this phenomenon. Pannus formation in diseased joints is accompanied by increased expression of ICAM-1,¹⁴ which can be upregulated by several cytokines including TNF and IL-1, and leads to the infiltration of mononuclear cells. In the present study, cA2 has been shown to block TNF-induced ICAM-1 (as well as E-selectin) expression by cultured human endothelial cells. The blocking by cA2 of ICAM-1 and E-selectin expression directly resulted in decreased adherence of human neutrophils to endothelial cell monolayers. Neutrophils are numerous in synovial fluid from RA patients and it is likely that their activation leads to further necrosis of tissue. It may therefore also be important that cA2 can block the priming of neutrophils by TNF. Although not investigated in this study, TNF also is involved in the induction of collagenase in synovial cells,³⁸ the inhibition of prostaglandin synthesis by articular chondrocytes³⁹ and the stimulation of bone resorption.⁴⁰ Moreover, it has been shown that the use of an anti-human TNF monoclonal antibody completely neutralizes development of disease in a human TNF transgenic model of arthritis.²⁰ The potential for reducing RA joint disease using an anti-TNF antibody approach has also been demonstrated in a murine model of collagen-induced arthritis.⁴¹ Mice were treated weekly with 10 mg/kg of anti-murine TNF antibody, with treatment beginning either before immunization with type II collagen or after immunization and onset of clinical arthritis. In both circumstances, the anti-TNF antibody reduced swelling of the paws and severity of disease by histopathological assessment of the arthritic joints.

While numerous cytokines such as TNF, IL-1, IL-6, GM-CSF and TGF- β have been identified in joint synovial fluids of patients with RA,¹⁵ recent studies by Feldmann and colleagues suggest that TNF may regulate the levels of these other cytokines.^{42, 43} Earlier studies have demonstrated that TNF is a potent inducer of IL-1 in endothelial cells⁴⁴ and monocytes,⁴⁵ as well as of IL-6⁴⁶ and GM-CSF.⁴⁷ Cells cultured from the synovial fluid from the diseased joints of RA patients continue to spontaneously produce bioactive IL-1. The addition of a polyclonal, neutralizing anti-human TNF antibody specifically reduced the levels of bioactive IL-1 β produced.⁴⁴ In a similar set of experi-

ments, levels of bioactive GM-CSF were shown to be significantly reduced by the addition of neutralizing anti-human TNF antibodies to the media of cultured synoviocytes from RA patients.⁴⁵ These results provide a clear rationale for the evaluation of anti-TNF therapy in RA, and results from initial studies to assess safety and efficacy in RA patients are encouraging.²⁴

TNF also has been cited as a potential mediator in several other autoimmune diseases. Elevated serum levels of TNF correlate with relapsing ulcerative colitis and chronic Crohn's disease⁴⁸ and TNF in the stool of patients with inflammatory bowel disease may be a marker which correlates with disease activity.⁴⁹ A temporary remission has been described in a Crohn's patient treated with cA2.²³ In this case report, a Crohn's patient who was unresponsive to conventional treatment received two doses of cA2 (10 mg/kg) spaced two weeks apart. Over a period of ten weeks after treatment, the patient gained weight and showed reduction in standard indexes of disease activity as well as complete endoscopic remission. Symptoms returned approximately 3 months after treatment. Anti-TNF antibodies have also been evaluated in experimental allergic encephalomyelitis (EAE), an autoimmune demyelinating disease in mice which mimics multiple sclerosis. Treatment of mice with neutralizing anti-TNF antibodies prevented transfer of EAE symptoms by a T cell clone⁵⁰ and delayed relapse caused by bacterial superantigen.⁵¹

It should be pointed out that the precise molecular role(s) of TNF in each of these disease states remains to be elucidated, and that further understanding of localized versus systemic effects of this cytokine is critical to the rational design of targeted anti-TNF therapy. This is particularly important as the role of TNF may be beneficial in certain disease states and under certain conditions.⁵² Highly specific, potent neutralizing agents such as cA2 provide a valuable tool for the elucidation of TNF biology in human disease, and may serve as new forms of therapeutic intervention in those cases where a causal relationship between aberrant TNF expression and disease pathology can be established. The results of the present study demonstrate the potential usefulness of cA2 and provide a rationale for its continued evaluation in human disease.

MATERIALS AND METHODS

Reagents

The monoclonal chimeric mouse/human anti-TNF IgG₁ (cA2) antibody was isolated from concentrated hybridoma cell supernatant by Protein A-Sepharose chromatography and ion exchange chromatography. Chimeric mouse/human 7E3 anti-platelet IgG₁, chimeric mouse/human 17-1A anti-tumour antigen IgG₁, and chimeric mouse/human MT-412 anti-CD4 IgG₁ were also purified by Protein A-Sepharose

chromatography and used as isotype-matched, irrelevant antibody controls. Lyophilized, carrier free recombinant human TNF (rhTNF) was obtained from Dainippon, Osaka, Japan; from Suntory, Osaka, Japan; and from Biosource, Camarillo, CA. The anti-E-selectin antibody H18/7 was a gift from Dr M. Bevilacqua, formerly at Brigham and Women's Hospital, Boston, MA. Anti-ICAM-1 antibody #11 was a gift from Dr G. Riethmuller, University of Munich, Germany. H18/7 and #11 antibodies were iodinated to a specific activity of 2-3 $\mu\text{Ci}/\mu\text{g}$ using Iodobeads (Pierce, Rockford, IL) as per the manufacturer's instructions. Human umbilical vein endothelial (HUVE) cells were obtained from Cell Systems, Kirkland, WA. The FS-4 fibroblast line was established and has been maintained at the New York University Medical Center. Recombinant constructs of the p55 and p75 human TNF receptors were a gift from Dr Bernard Scallan, Department of Molecular Biology, Centocor, Inc. Briefly, the p55-sf2 construct contains the extracellular domain of human p55 fused to eight amino acids of human antibody J sequence followed by all three constant domains of human IgG1. It is disulfide bonded to a human kappa light chain constant region. The p75P-sf2 construct is the same as p55-sf2 except it contains a truncated form of the extracellular domain of human p75. Both constructs were purified from cell culture supernatant by Protein A affinity chromatography.

Cell culture

Human umbilical vein endothelial (HUVE) cells were grown in complete HUVE medium (Cell Systems, Kirkland, WA) containing 15% fetal bovine serum (FBS; Hyclone, Logan UT) supplemented with growth factor (CS-HBGF-1/H; Cell Systems, Kirkland, WA) on T-75 tissue culture flasks (Corning, Corning, NY). All plasticware used for HUVE cell culture was coated first with attachment factor (Cell Systems, Kirkland, WA) by wetting the surface with attachment factor and removing the excess fluid prior to introducing cells. Cells were serially passaged by splitting 1:3 in the same medium every 3-5 days and all assays were performed on cells at passages 2-4.

FS-4 fibroblasts were grown in modified Eagle's medium (MEM) containing 5% FBS in T-75 flasks. Cells were serially passaged by splitting 1:5 in the same medium every 2 weeks; cells at passage level 13 to 15 were used in the experiments.

Mitogenesis assay

FS-4 fibroblasts were seeded at 8×10^3 cells/well in a 96-well tissue culture plate in MEM containing 5% FBS and cultured for 18 h at 37°C. Recombinant human TNF was diluted in complete MEM to final concentrations of 0, 0.1, 0.5 and 2 ng/mL in the absence or presence of 4 $\mu\text{g}/\text{mL}$ test antibody. The samples were preincubated for 20 min at room temperature, then 0.1 mL was added to quintuplicate wells and incubated at 37°C for 5 days. The cells were rinsed with phosphate buffered saline (PBS) pH 7.2, then fixed by adding 50 $\mu\text{L}/\text{well}$ of 10% formalin in PBS for 15 min at room temperature. The fixed cells were then stained with 50 $\mu\text{L}/\text{well}$ of 0.05% naphthol blue black in 9% acetic acid, 0.1 M sodium acetate for 30 min at room temperature. The cells were then rinsed thoroughly with distilled water, and the

bound dye was eluted with 150 $\mu\text{L}/\text{well}$ of 50 mM NaOH. Absorbance of the eluted dye was determined at 630 nm.

Assay for IL-6

FS-4 fibroblasts were seeded at 2×10^4 cells/well in a 96-well tissue culture plate in MEM containing 5% FBS and cultured overnight at 37°C. Recombinant human TNF was diluted to final concentrations of 0, 0.3, 1.5 and 7.5 ng/mL in the absence or presence of 4 $\mu\text{g}/\text{mL}$ test antibody. The samples were preincubated for 20 min at room temperature, then 0.1 mL was added to duplicate culture wells and the incubation continued for 18 h. The cell supernatants from duplicate wells were pooled and stored at -20°C. The amount of IL-6 present in each sample was determined using an ELISA-based plate assay (Quantikine IL-6, R&D Systems, Minneapolis, MN) as described by the manufacturer.

Procoagulant activity assay (PCA)

The PCA assay was performed on HUVE cells plated at 1.5×10^5 cells/well in 24-well tissue culture plates (Falcon) coated with attachment factor. Confluent monolayers were washed three times with 500 $\mu\text{L}/\text{well}$ of RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 1% FBS and 0.3 mg/mL L-glutamine (JRH Biosciences, Lenexa, KS). Antibody samples were tested at 3.3, 1.1, 0.37 and 0.12 $\mu\text{g}/\text{mL}$ for neutralization of rhTNF at a final concentration of 25 ng/mL. Medium alone (no rhTNF) and medium plus 10 $\mu\text{g}/\text{mL}$ cA2, were used as negative controls while medium containing rhTNF at 25 ng/mL (no antibody) was used as a positive control. All dilutions were prepared in complete RPMI medium and were preincubated for 30 min at room temperature prior to incubation with the endothelial cells. Test solutions were dispensed into duplicate wells, 500 $\mu\text{L}/\text{well}$, and incubated for 4 h at 37°C. The test solutions were removed with gentle aspiration and the cells were washed as described previously. Three hundred microliters of complete RPMI were dispensed into each well and the plates were immediately frozen at -70°C. Cell lysates were prepared by thawing the plates at room temperature, resuspending all cell debris, and freezing and thawing each plate two more times. The plasma clotting assay was performed after equilibrating all reagents at 37°C. Clotting was initiated by mixing 0.1 mL of fresh, citrated human plasma, 0.1 mL of cell lysate and 0.1 mL of 30 mM CaCl_2 in a glass tube and incubating at 37°C. The time required for the clot to form (by visual inspection) was recorded and the mean and standard deviation from duplicate cell lysates were calculated.

Adhesion protein assays

The E-selectin and ICAM-1 assays were performed on HUVE cells plated at 5×10^4 cells/well in 96-well tissue culture plates (Costar 3596, Cambridge, MA) coated with attachment factor. Confluent monolayers were gently washed twice using a multi-channel pipettor with 150 $\mu\text{L}/\text{well}$ of HUVE medium. Twofold serial dilutions of cA2 or c17-1A were prepared in medium containing 10 ng/mL rhTNF. Medium alone was used as a negative control while medium containing 10 ng/mL rhTNF was used as a positive control. Test solutions were dispensed into triplicate wells, 100 $\mu\text{L}/\text{well}$.

well, and incubated for 4 h (E-selectin assay) or 23 h (ICAM-1 assay) at 37°C. The test solutions were removed with a multi-channel pipettor and the iodinated probes were added. Expression of adhesion protein was detected by incubating the cells for 2 h at room temperature with ^{125}I anti-E-selectin antibody H18/7 F(ab')₂ or ^{125}I -anti-ICAM-1 #11 IgG diluted to 10 µg/mL in complete HUVE medium (300000 cpm/100 µL/well). The cells were then washed 4× with RPMI-1640 containing 10% FBS, and the well contents solubilized and counted for ^{125}I in a gamma counter.

Neutrophil adhesion and priming assays

Neutrophils were isolated from 100 mL of fresh human blood drawn into heparin. Ten millilitres of blood were layered onto 5 mL of Mono-poly resolving medium (Flow Labs, McLean, VA) in a 15 mL conical centrifuge tube and centrifuged at $750 \times g$ for 30 min at room temperature. Additional centrifugation for 30 min at $900 \times g$ was generally required to completely separate the bands of cells. The top band containing T and B cells was discarded and the lower band containing polymorphonuclear cells was collected. The neutrophils were washed with Hank's buffered saline (without magnesium and calcium) and were resuspended in RPMI-1640/10% FBS.

For the adhesion assay, HUVE cells grown in 24-well tissue culture plates were stimulated for 4 h at 37°C with rhTNF (250 ng/mL), IL-1 α (40 units/mL; Genzyme, Boston, MA) or *E. coli* J5 LPS (10 ng/mL; List Biological Laboratories, Campbell, CA) in RPMI-1640/10% FBS containing the indicated concentration of test antibody, or with medium alone. The HUVE monolayers, containing about 3×10^5 cells per well, were then washed once and overlaid with 0.1 mL of RPMI-1640/10% FBS containing 1×10^6 neutrophils. After incubation for 45 min at 37°C, the monolayers were gently washed three times with RPMI-1640/10% FBS and then solubilized with 0.25 mL of 50 mM potassium phosphate pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The solubilized monolayer samples were then quantitated using the myeloperoxidase assay in which known quantities of isolated neutrophils were processed in the same manner and used to generate a standard curve. Standard samples were analysed in duplicate while test samples were tested in triplicate. Reagents were added to the wells of a 96-well microtiter plate in the following order: 55 µL of 80 mM potassium phosphate pH 5.4; 15 µL of sample, standard or blank; 20 µL of 0.3 mM hydrogen peroxide in 80 mM potassium phosphate pH 5.4; 10 µL of 16 mM 3,3',5,5'-tetramethylbenzidine in N,N'-dimethylformamide. The plate was incubated for 15 min at room temperature and the reaction stopped by adding 100 µL of 1 M phosphoric acid per well. The optical density of each well was read at 450 nm. Photomicroscopy of HUVE cell monolayers at 100× magnification was performed using a phase contrast Nikon IMT-2 inverted research microscope.

For neutrophil oxidative burst assays, 2.5×10^6 cells/mL were primed with 2.0 ng/mL rhTNF in the presence of the indicated concentration of test antibody for 60 min at 37°C. Primed cells were activated (or mock-activated) with 0.1 µM FMLP (Sigma, St. Louis, MO) for 10 min at 37°C in the presence of 1 mg/mL cytochrome C (Sigma, St. Louis, MO).

The cells were then microcentrifuged for 5 min and the OD of the supernatants read at 550 nm. Duplicate samples containing 10 µg/mL superoxide dismutase (SOD; Sigma) were run in parallel and the background OD obtained subtracted from samples without SOD. The results were converted to nM superoxide ion using the extinction coefficient for (reduced cytochrome c) – (oxidized cytochrome c) for a 3 mm path length.³³

Receptor binding assays

^{125}I -rhTNF binding to U937 membranes was performed using a New England Nuclear (Boston, MA) ligand binding kit. Briefly, 45 pM of ^{125}I -rhTNF (40–50 µCi/µg), the indicated concentrations of test antibody and U937 membranes provided, were incubated as described in the manufacturer's instructions in a final volume of 250 µl for 3 h on ice. Membrane bound tracer was separated from free tracer by vacuum filtration over GF/C filters. The filters were washed 2×4 mL with the wash buffer provided. Data were expressed as the mean \pm SEM of three separate experiments and graphed as percent of control ^{125}I -rhTNF binding in the absence of antibody (approximately 1100 cpm). Binding of tracer in the presence of 10 µg/mL of a negative control antibody was $96\% \pm 1\%$.

To assess ^{125}I -rhTNF binding to recombinant constructs of the p55 and p75 cellular receptors for TNF, 50 µL of a 5 µg/mL solution of either p55 or p75 receptor fusion proteins in PBS was incubated on polystyrene 96-well plates for 1 h at 37°C. The wells were washed and blocked for 1 h at 37°C with PBS containing 1% BSA. Equal volumes (25 µL each) of serially diluted cA2 in PBS/1% BSA and $2 \times ^{125}\text{I}$ -rhTNF (final concentration = 4 ng/mL) were added to duplicate wells. Plates were incubated for 1 h at 37°C, washed 2×200 µL with PBS and the radioactivity bound was counted in a gamma counter. Binding of tracer in the presence of 10 µg/mL negative control antibody was 2270 cpm.

Transgenic mouse protection model

Tg211 transgenic mice were bred as previously described¹⁹ and randomly divided into groups of 15 animals each. These groups received twice-weekly intraperitoneal injections of 10 µL per gram average body weight to achieve a final dose of 0.5, 2 and 8 mg/kg cA2 IgG. A fourth group received 8 mg/kg of an isotype-matched control antibody. Investigators (LP and GK) performing the experiment at the Hellenic Pasteur Institute were blinded with respect to the drug each treatment group received during the course of the study. Injections of test antibody were initiated when the mice reached 3 weeks of age, and the study was terminated after 8 weeks of treatment. Average weight and mortality in each group was recorded weekly.

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